



Assessing the agricultural reuse of the digestate from microalgae anaerobic digestion and co-digestion with sewage sludge

Maria Solé-Bundó^a, Mirko Cucina^{a, b}, Montserrat Folch^c, Josefina Tàpias^c, Giovanni Gigliotti^b, Marianna Garfí^a, Ivet Ferrer^{a, *}

^a GEMMA - Environmental Engineering and Microbiology Research Group, Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya-BarcelonaTech, c/ Jordi Girona 1-3, Building D1, E-08034 Barcelona, Spain

^b Department of Civil and Environmental Engineering, University of Perugia, Borgo XX Giugno 74, 06124 Perugia, Italy

^c Department of Natural Products, Plant Biology and Soil Science, School of Pharmacy, University of Barcelona, Avda. Joan XXIII s/n, E-08028 Barcelona, Spain

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ABSTRACT

Microalgae anaerobic digestion produces biogas along with a digestate that may be reused in agriculture. However, the properties of this digestate for agricultural reuse have yet to be determined. The aim of this study was to characterise digestates from different microalgae anaerobic digestion processes (i.e. digestion of untreated microalgae, thermally pretreated microalgae and thermally pretreated microalgae in co-digestion with primary sludge). The main parameters evaluated were organic matter, macronutrients and heavy metals content, hygienisation, potential phytotoxicity and organic matter stabilisation. According to the results, all microalgae digestates presented suitable organic matter and macronutrients, especially organic and ammonium nitrogen, for agricultural soils amendment. However, the thermally pretreated microalgae digestate was the least stabilised digestate in comparison with untreated microalgae and co-digestion digestates. In vivo bioassays demonstrated that the digestates did not show residual phytotoxicity when properly diluted, being the co-digestion digestate the one which presented less phytotoxicity. Heavy metals contents resulted far below the threshold established by the European legislation on sludge spreading. Moreover, low presence of *E. coli* was observed in all digestates. Therefore, agricultural reuse of thermally pretreated microalgae and primary sludge co-digestate through irrigation emerges a suitable strategy to recycle nutrients from wastewater.

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1. Introduction

Microalgae-based wastewater treatment systems represent a cost-effective alternative to conventional activated sludge systems. The major advantage is that mechanical aeration is not required, since oxygen is provided by microalgae photosynthesis. Moreover, microalgae cultures are capable of removing nutrients (N, P) from wastewater by means of different mechanisms, such as assimilation or precipitation (Rawat et al., 2011). Furthermore, these systems can also combine wastewater treatment and bioenergy production if harvested microalgal biomass is downstream processed. In particular, anaerobic digestion is one of the most well-known processes to valorise organic waste generated in a wastewater treatment plant. Over the last decades, several studies on biogas production from microalgae have been carried out (Uggetti et al., 2017). They have demonstrated that some microalgae species have a resistant cell wall, which may hamper their bioconversion into methane. Microalgae cell wall disruption could be enhanced by applying pretreatment methods, being the most suitable those pretreatments with low energy demands (Passos et al., 2014). Besides, in the context of microalgae grown in wastewater, co-digestion of microalgae with sewage sludge is a prof-

itable strategy, since the sludge is generated in the same process chain (Uggetti et al., 2017). This could optimise waste management and increase the organic loading rate of the digester (Mata-Alvarez et al., 2014).

Apart from biogas, microalgae anaerobic digestion also produces a digestate that can be reused in agriculture. Even though several studies have pointed out the necessity of recycling nutrients through digestate reuse to improve the sustainability of biogas production from microalgae (Collet et al., 2011), the properties of microalgae digestate for agricultural reuse have yet to be characterised. In general, anaerobic digestates have proper chemical properties for agricultural reuse (Rowell et al., 2001). For instance, they are rich in ammonia nitrogen, readily available for plant uptake, and other macronutrients such as phosphorus and potassium (Teglia et al., 2011a). However, depending on digestates properties, their reuse could be more addressed to improve or maintain the physico-chemical or biological properties of soils (soil amendment) or to boost the plants growing (fertilisers). In the first case, digestates with high organic matter, organic carbon and organic nitrogen content are preferred, while digestates with important mineral fractions have a higher potential for application as fertiliser (Nkoa, 2014).

Anaerobic digestion is often designed to achieve the maximum energy production, leading to a low stabilisation of the organic matter of the feedstock. As a consequence, digestates may be characterised by a high labile organic matter content and, thus, their agricultural

* Corresponding author.

Email address: ivet.ferrer@upc.edu (I. Ferrer)

reuse may face agronomic and environmental issues. In fact, it is known that by adding low-stabilised organic matter the soil microbial activity may be excessively stimulated. Indeed, it can produce high CO₂ fluxes from the soil, soil oxygen consumption with sequential nitrogen losses, and phytotoxicity phenomena (Pezzolla et al., 2013; Abdullahi et al., 2008). In addition, the digestate composition can highly vary depending on the feedstock or anaerobic digestion operating conditions. Even the application of a pretreatment on the feedstock previous to anaerobic digestion can influence the final composition of the digestate (Monlau et al., 2015a). Thus, the characterisation of a digestate before evaluating its potential applications is convenient.

When characterising new digestates, particular attention should be addressed to the macronutrients content, potential phytotoxicity and stabilisation of the organic matter. In vivo bioassays are useful to assess the potential phytotoxicity (Alburquerque et al., 2012; Zucconi et al., 1985). The quantification of CO₂ emissions and the water extractable organic matter (WEOM) in digestate amended soils are suitable strategies to assess organic matter stabilisation (Pezzolla et al., 2013; Said-Pullicino and Gigliotti, 2007). On the other hand, land application of anaerobic digestates may also introduce physical, chemical and biological contaminants into soils which may be up-taken by crops and endanger their long-term agricultural activity (Nkoa, 2014). For instance, European legislation on sewage sludge spreading (CEC, 1986) mainly regulates the heavy metals content in digestates to avoid their accumulation in amended soils. However, a more recent European Directive draft (CEC, 2003b) also proposes restrictions on the occurrence of bio-accumulative organic compounds and their hygienisation before being spread on soils. Consequently, the presence of these contaminants in digestates should be assessed if they are going to be reused in agricultural soils.

The aim of this study was to characterise for the first time the quality of microalgal digestates for agricultural reuse. To this end, the effluents from three different anaerobic digesters fed by untreated microalgae, thermally pretreated microalgae and thermally pretreated microalgae in co-digestion with primary sludge were analysed. The main parameters evaluated were organic matter, macronutrients and heavy metals content, hygienisation, potential phytotoxicity and organic matter stabilisation.

2. Material and methods

2.1. Digestate origin and sampling

The microalgal biomass used in this study consisted of a microalga-bacteria consortia grown in a pilot raceway pond that treated wastewater from a municipal sewer, as described by Passos et al. (2015). Microalgal biomass was harvested from secondary settlers and gravity thickened in laboratory Imhoff cones at 4 °C for 24 h. The pilot plant was located at the laboratory of the GEMMA research group (Barcelona, Spain). According to optic microscope examinations (Motic BA310E, equipped with a camera NiKon DS-Fi2), predominant microalgae were *Chlorella* sp. and diatoms (Fig. 1).

In order to improve microalgae biodegradability, a part of the harvested and thickened biomass was thermally pretreated at 75 °C for 10 h, as suggested by Passos and Ferrer (2014). The pretreatment of microalgal biomass was carried out in glass bottles with a total volume of 250 mL and a liquid volume of 150 mL, which were placed in an incubator under continuous stirring at 75 °C for 10 h. Untreated (control) and pretreated microalgae were digested in lab-scale reactors under mesophilic conditions. Furthermore, the anaerobic co-di-

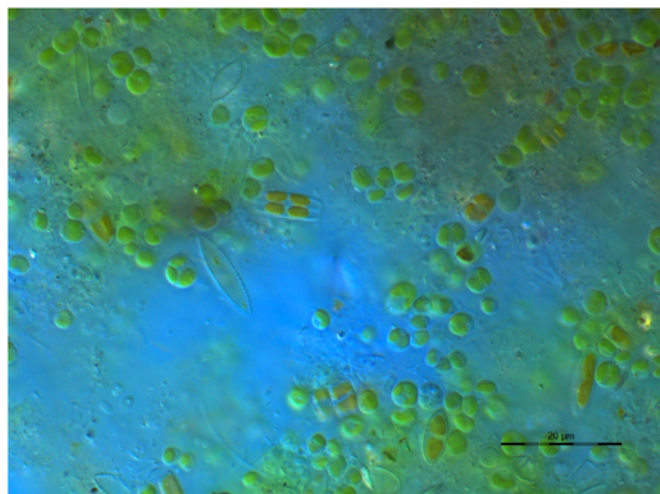


Fig. 1. Microscopic image of microalgal biomass mainly composed by *Chlorella* sp. and diatoms.

gestion of pretreated microalgal biomass with primary sludge (25%–75% VS, respectively) was also evaluated. The thickened primary sludge was collected in a municipal wastewater treatment plant near Barcelona.

Thus, the following effluents from microalgae anaerobic digestion were analysed:

- Digester 1 (D1): microalgal biomass;
- Digester 2 (D2): thermally pretreated microalgal biomass;
- Digester 3 (D3): co-digestion of pretreated microalgal biomass and primary sludge.

Anaerobic reactors (1.5 L) were operated on a daily feeding basis, where same volume was purged from and added to digesters using plastic syringes. Operation conditions of the reactors and feedstock characteristics are shown in Table 1. Digestate samples were analysed weekly over a period of 11 weeks of stable reactors operation. Physico-chemical properties were analysed during 11 weeks (n = 11) while macronutrients and pathogens were analysed during the last 6 weeks (n = 6) and the heavy metals during the 3 last weeks (n = 3).

Table 1
Main parameters of the anaerobic digestion and feedstock properties.

	Digester 1 (D1): microalgae	Digester 2 (D2): pretreated microalgae	Digester 3 (D3): co-digestion
Operation conditions			
Temperature (°C)	36.2 ± 1.1	36.6 ± 1.8	35.7 ± 1.8
OLR (gVS/L·day)	0.83 ± 0.04	0.82 ± 0.02	0.83 ± 0.01
HRT (days)	30	30	30
Feedstock			
Composition (% VS)	100% MB	100% P-MB	25% P-MB + 75% PS
TS (%)	3.9 ± 0.4	3.7 ± 0.3	3.7 ± 0.4
VS (%)	2.5 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
VS/TS (%)	66 ± 5	66 ± 6	66 ± 8
COD (g/L)	43.4 ± 8.1	44.0 ± 7.0	48.1 ± 8.0

Note: OLR = organic loading rate, HRT = hydraulic retention time, TS = total solids, VS = volatile solids, COD = chemical oxygen demand, MB = microalgal biomass, P-MB = pretreated microalgal biomass, PS = primary sludge.

Pretreatment conditions: 75 °C, 10 h.

2.2. Digestate characterisation

2.2.1. Physicochemical properties and macronutrients

Total solids (TS), volatile solids (VS), total chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN) were analysed according to Standard Methods (APHA, 2005). Ammonium nitrogen ($\text{NH}_4^+\text{-N}$) was measured according to the Solorzano method (Solorzano, 1969). Volatile fatty acids (VFA) concentrations were measured by injecting 1 μL of centrifuged (4200 rpm for 8 min) and filtered samples (0.2 μm) into an Agilent 7820A GC after sulphuric acid and diisopropyl ether addition. The GC was equipped with an auto-sampler, flame ionisation detector and a capillary column (DP-FFAB Agilent 30 m \times 0.25 mm \times 0.25 μm), and operated at injector and detector temperatures of 200 and 300 $^\circ\text{C}$, respectively, with helium as carrier gas. Electric conductivity (EC) was determined with a Crison EC-Meter GLP 31+ and pH with a Crison Portable 506 pH-meter. Total organic carbon (TOC) and total nitrogen (TN) were measured using an automatic analyzer (aj- Analyzer multi N/C 2100S). TOC was analysed with an infrared detector (NDIR) according to combustion-infrared method of Standard Methods (APHA, 2005) by means of catalytic oxidation at 800 $^\circ\text{C}$ using CeO_2 as catalyst. Following, a solid-state chemical detector (ChD) was used to quantify TN as NO_x . Phosphorous was determined by means of Olsen-P modified method (Watanabe and Olsen, 1965). Ca^{+2} and Mg^{+2} were analysed by EDTA titrimetric method after ammonium acetate extraction (1 N at pH 7), while Na^+ and K^+ were determined by flame photometric method after ammonium acetate extraction (1 N at pH 7) (MAPA, 1994). Dewaterability was evaluated by means of the capillary suction time (CST) test (Triton Electronics Ltd.).

2.2.2. Heavy metals

In order to determine the heavy metals concentration, samples were dried at 100 $^\circ\text{C}$ during 24 h. After HCL-HNO_3 (3:1, v/v) digestion (200 $^\circ\text{C}$, 15 min) of dry digestate, Cd, Cr, Cu, Hg, Ni, Pb and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer Elan 6000).

2.2.3. Pathogens

Escherichia coli (*E. coli*) was determined according to Standard Methods (APHA, 2005). The *E. coli* ChromID™ Coli (COLI ID-F) used in this study was supplied by Biomérieux and the culture medium was m-coliBlue24® from Difco.

2.3. Organic matter stabilisation

2.3.1. Soil incubation procedure

Organic matter stabilisation from digestates was evaluated through a microcosm soil experiment. Fresh digestates were used to amend an agricultural soil (soil chemical characterisation not shown), using a digestate dose according to the limits prescribed by the European Nitrates Directive (CEC, 1991) for the protection of groundwater against pollution caused by nitrates. Specifically, digestate application doses were calculated to apply 170 kg N ha $^{-1}$. 200 g of soil (dry matter) were amended and placed in an incubation chamber (20 \pm 2 $^\circ\text{C}$) for 30 days at 70% of the water holding capacity.

2.3.2. CO $_2$ emissions evaluation

CO $_2$ emissions resulting from the organic matter mineralization were measured after 0, 2, 5, 8, 12, 20 and 30 days of amending, using

an alkaline-trap and subsequent titration. At the same time, 10 g (fresh weight) of soil were collected and air-dried for the WEOM determination.

2.3.3. Water extractable organic matter determination

The WEOM was analysed both in the digestates and amended soils. Fresh digestate samples were centrifuged at 4200 rpm for 6 min and filtered through a 0.45 μm membrane filter (GVS). Soil WEOM was extracted from the dry soil samples with deionised water (solid to water ratio of 1:10 w/w) for 24 h. The suspensions were then centrifuged at 4200 rpm for 6 min and filtered through a 0.45 μm membrane filter. Water Extractable Organic Carbon (WEOC) concentration in the filtrates was then measured by an automatic analyzer (Analytic Jena-Analyzer multi N/C 2100S) and the WEOM was calculated according the following equation (Pribyl, 2010):

$$\text{WEOM} = \text{WEOC} \cdot 2.0$$

2.4. Potential phytotoxicity

2.4.1. Seed germination bioassay

To evaluate the germination index (GI), a modified phytotoxicity test employing seed germination was used (Zucconi et al., 1985). Pure digestates together with three dilutions (0.1%, 1% and 10% v/v in deionised water) were used as germination media. A filter paper placed inside a 9 cm diameter Petri dish was wetted with 1 mL of each germination solution and 10 *Lepidium sativum* L. seeds were placed on the paper. 100% deionised water was used as a control. Five replicates were set out for each treatment. The Petri dishes, closed with plastic film to avoid moisture loss, were kept in the dark for 2 days at 20 $^\circ\text{C}$. After the incubation period, the number of germinated seeds and the primary root length were measured. The GI was expressed as a percentage of the control.

2.4.2. Plant growth bioassay

To evaluate the influence of digestate on plant biomass accumulation, a modified phytotoxicity test employing plant growth was used (Albuquerque et al., 2012). Plastic seedbeds made of 12 cells (50 mL/cell with a drainage hole in the bottom) were used for the experiment, after filling them with commercial perlite (2–3 mm diameter). Seedbeds were placed 24 h in a vessel (20 \times 15 \times 5 cm) containing 500 mL of deionised water to reach the saturation of the substrate. Then, 5 seeds of *Lepidium sativum* L. were sown in each cell. After the 3 days needed for the germination and seedlings occurrence, 32 seedlings were left in each seedbed and deionised water was replaced by 500 mL of the digestate dilutions to be tested (0.1%, 1% and 10% v/v). Pure digestates were not tested in this case, since no germination was observed in the germination test. One seedbed was used as a control, leaving 100% deionised water as growth media. During all the experiment, the vessels were placed in environmental controlled conditions (25 \pm 2 $^\circ\text{C}$, daily photoperiod of 14 h). At the end of the experiment, after 10 days from the replacement of the growth media, seedlings survived were harvested and their total dry mass (TS) was determined after drying at 105 $^\circ\text{C}$. The growth index (GrI) was calculated for each digestates as the percentage of the control (distilled water). The whole experiment was replicated three times.

3. Results and discussion

3.1. Physico-chemical characterisation

All the digestates analysed presented low dry matter content (~ 3% TS) (Table 2) and can be considered as liquid products. To ease their management, these digestates could be directly spread on soils in nearby areas. However, if transportation/distribution was required, a dewatering process to reduce the moisture content would be recommended. If we look at the CST measurements, which estimate the ability of each digestate to release water (Gray, 2015), we can see how microalgae digestates presented poor dewaterability (25 and 28 s·gTS⁻¹·L for D1 and D2, respectively), while these results were consistently improved by the co-digestion of primary sludge (8 s·gTS⁻¹·L) (Table 2). This is due to the higher dewaterability of primary sludge digestate with respect to microalgae digestate.

On the other hand, the measured pH presented slightly-alkaline values in all digestates (> 7.0). Among them, pretreated microalgae digestate (D2) presented the highest pH value, which can be attributed to the higher concentration of NH₄⁺-N released from proteins during the thermal pretreatment (Passos and Ferrer, 2014). However, all pH values are compatible with the common pH on soils and therefore, their application should not affect the soil pH.

Other factors that may cause an impact on soils after digestate spreading are the EC and VFA's content, since phytotoxicity effects have been correlated to both parameters (Albuquerque et al., 2012; Di Maria et al., 2014). Although EC was moderate in all digestates (5.9–8.2 dS·m⁻¹), the digestate from the co-digestion showed the lowest value. Consequently, it would cause less impact on soil. Besides, all digestates showed low VFA's concentrations (Table 2). Again, the lowest value was found in the co-digestion digestate (10 mg·COD·eq·L⁻¹). This indicates that the anaerobic digestion process results in a more stabilised digestate when pretreated microalgae are co-digested with the primary sludge.

3.2. Organic matter and fertiliser properties

The three digestates had moderate organic content due to organic matter mineralization during the anaerobic digestion process. While the two microalgae digestates presented a similar VS/TS ratio of

53–54%, the percentage of organic matter in the co-digestion digestate was lower (47%) due to the higher mineralization of primary sludge, which is a more readily biodegradable substrate than microalgae. In fact, the percentage of organic matter in digestates is highly dependent on the type of substrate and the operating conditions of anaerobic reactors (Monlau et al., 2015b). For instance, Teglia et al. (2011a) compared digestates from different origins and found that digestates from agri-food industries showed higher organic matter content than digestates from sewage treatment plants. The results obtained in this study are in accordance with those from similar microalgae anaerobic digestion processes (Passos and Ferrer, 2014, 2015).

Several studies have shown that anaerobic digestates can be as effective as mineral fertilisers (Nkoa, 2014). To assess the fertiliser properties of the microalgae digestates, the macronutrients content was here evaluated (Table 3). The main nutrient present in all digestates was nitrogen. Even so, the nitrogen content of microalgae digestates (both untreated and thermally pretreated) was significantly higher than the co-digestion digestate (39–42%), showing values of 80 g·kg TS⁻¹ and 56 g·kg TS⁻¹, respectively. Microalgae digestates presented similar nitrogen values compared to those from farm-byproducts that are frequently applied as nitrogen suppliers on soils (Albuquerque et al., 2012; Zucconi et al., 1985). Moreover, the nitrogen content was much higher than the common values found in sewage sludge digestates (36–40 g·kg TS⁻¹) (Di Maria et al., 2014; Gell et al., 2011), even in the co-digestion digestate. The highest concentration of NH₄⁺-N was found in the pretreated microalgae digestate. However, the NH₄⁺-N/TKN ratio only varied from 30.9 to 33.8% among all digestates, presenting all of them a similar soluble mineral nitrogen fraction. This means that the organic nitrogen fraction is predominating in all digestates, so they should be used as soil amendment rather than fertiliser (Teglia et al., 2011b). As expected, the digestates also showed low C/N ratios around 3 (Table 2). These values are within the typical range for other digestates as sewage sludge, poultry slurry or pig slurry (Albuquerque et al., 2012; Gutser et al., 2005). Unfortunately, with low C/N ratios, N is present in excess and it can be lost by ammonia volatilization or leaching (Bernal

Table 2

Main physico-chemical properties and organic matter of the three microalgae digestates analysed (mean ± SD; n = 11, except for TOC and TN (n = 3)).

Parameter	Units	Digestate D1: microalgae	Digestate D2: pretreated microalgae	Digestate D3: co-digestion
pH	–	7.35 ^a ± 0.11	7.55 ^b ± 0.08	7.30 ^a ± 0.15
EC	dS·m ⁻¹	7.0 ^b ± 0.7	8.2 ^a ± 0.3	5.9 ^c ± 0.4
TS	g·g ⁻¹ , %	3.0 ^a ± 0.1	2.9 ^a ± 0.2	3.0 ^a ± 0.2
VS	g·g ⁻¹ , %	1.6 ^b ± 0.1	1.5 ^b ± 0.1	1.4 ^a ± 0.1
VS/TS	%	54 ^b ± 2	53 ^b ± 1	47 ^a ± 2
COD	g·L ⁻¹	26 ^a ± 2	25 ^a ± 2	24 ^a ± 1
TOC	g·L ⁻¹	7.6 ± 0.1	6.4 ± 0.0	6.1 ± 0.1
TN	g·L ⁻¹	2.4 ± 0.0	2.2 ± 0.1	1.9 ± 0.1
C/N	–	3.17	2.98	3.27
VFA	mgCOD·eq·L ⁻¹	100 ^a ± 138	270 ^a ± 365	10 ^a ± 25
CST	s	795 ^b ± 71	919 ^b ± 122	272 ^a ± 21
	s·gTS ⁻¹ ·L	25 ^b ± 3	28 ^b ± 4	8 ^a ± 1

Note: TS = total solids, VS = volatile solids, COD = chemical oxygen demand, TOC = total organic carbon, TN = total nitrogen, C/N = Carbon-Nitrogen ratio, VFA = volatile fatty acids, CST = capillary suction time.

^{a,b,c}: Letters indicate a significant difference between digestates at a level of $p < 0.05$ after Tukey's test.

Table 3

Macronutrients characterisation of the three digestates analysed (mean ± SD, n = 6).

Parameter	Units	Digestate D1: microalgae	Digestate D2: pretreated microalgae	Digestate D3: co-digestion
TKN	gN·L ⁻¹	2.4 ^a ± 0.1	2.3 ^a ± 0.1	1.7 ^b ± 0.0
	gN·kg TS ⁻¹	79.8 ^a ± 4.0	80.6 ^a ± 2.2	56.0 ^b ± 1.1
NH ₄ ⁺ -N	gN·L ⁻¹	0.7 ^b ± 0.1	0.8 ^b ± 0.1	0.5 ^a ± 0.1
NH ₄ ⁺ -N/TKN	%	30.9	33.8	32.5
P	gP ₂ O ₅ ·L ⁻¹	0.25 ^b ± 0.02	0.27 ^b ± 0.02	0.21 ^a ± 0.03
	gP·kg TS ⁻¹	3.6 ^b ± 0.3	3.9 ^b ± 0.2	3.2 ^a ± 0.5
K	gK ₂ O·L ⁻¹	0.17 ^b ± 0.03	0.19 ^b ± 0.02	0.08 ^a ± 0.03
	gK·kg TS ⁻¹	4.8 ^b ± 0.8	5.2 ^b ± 0.7	2.2 ^a ± 1.0
Ca	gCaO·L ⁻¹	0.43 ^a ± 0.13	0.37 ^a ± 0.10	0.54 ^b ± 0.07
	gCa·kg TS ⁻¹	10.2 ^a ± 3.1	8.9 ^a ± 2.4	13.4 ^b ± 1.7
Mg	gMgO·L ⁻¹	0.18 ^a ± 0.09	0.21 ^a ± 0.09	0.17 ^a ± 0.10
	gMg·kg TS ⁻¹	3.6 ^a ± 1.8	4.2 ^a ± 1.8	3.6 ^a ± 2.0
Na	gNa ₂ O·L ⁻¹	0.40 ^b ± 0.05	0.38 ^b ± 0.06	0.32 ^a ± 0.03
	gNa·kg TS ⁻¹	10.0 ^b ± 1.3	9.4 ^b ± 1.4	8.1 ^a ± 0.8

Note: TKN = total Kjeldahl nitrogen.

^{a,b}: Letters indicate a significant difference between digestates at the level of $p < 0.05$ after Tukey's test.

et al., 2009). In order to increase the carbon content in microalgae digestates, they could be co-digested with other carbon rich substrates, like waste paper (Yen and Brune, 2007).

Moderate quantities of P and K⁺ were also found in all the digestates (Table 3). P content was slightly higher in microalgae digestates (D2 and D3) compared to the digestate obtained by the co-digestion (3.6–3.9 and 3.2 g P·kg TS⁻¹, respectively). On the other hand, the content of K⁺ of the microalgae digestates was 2-fold higher compared to the digestate obtained by the co-digestion (4.8–5.2 and 2.2 g K·kg TS⁻¹, respectively). Conversely to nitrogen, no significant differences were found between P and K⁺ contents of microalgae and sewage sludge digestates. In particular, literature reported values from 2.2–3.0 g K·kg TS⁻¹ and 3.2–3.8 g P·kg TS⁻¹ in sewage sludge digestates (Di Maria et al., 2014; Gell et al., 2011; Tambone et al., 2010), which fall within the range of the co-digestion digestate analysed in the present study. Ca²⁺, Mg²⁺ and Na⁺ presented similar concentrations in all the cases. This can be attributed to the composition of the wastewater treated in both systems where microalgae and primary sludge were obtained, which came from the same water source. The content of salts should be carefully analysed when applying the digestates to the soils to avoid their salinization, especially the presence of Na⁺ (Daliakopoulos et al., 2016).

On the whole, microalgae digestates could especially contribute to nitrogen supply on soils. However, with a moderate NH₄⁺-N/TKN ratio (<35%) their use should be addressed as soil amendment rather than direct biofertiliser. Indeed, the digestates nutrients content was lower than those recommended by the standards of European countries that have regulated the commercial uses of liquid fertilisers (CEC, 2003a). Conversely, their organic matter content and their high mineral and organic nitrogen content make them suitable for land spreading. Nonetheless, the stability of organic matter and potential toxicity of digestates must be taken into account, along with their potential risks on soil contamination. These issues are analysed and discussed in the following sections.

3.3. Stabilisation of the organic matter

Fig. 2a shows the CO₂ emissions measured from the digestate amended soils studied in the microcosm experiment. Whereas the control (un-amended soil) showed moderately constant emission rates throughout the incubation period, the addition of digestates increased the CO₂ fluxes with respect to the control, particularly in the first days after amendment. Similar results were obtained by other authors after amending soils with anaerobic digestate and compost (Alluvione et al., 2010; Pezzolla et al., 2013). The highest emission rates were observed immediately after applying the digestates for the soils treated with pretreated microalgae (D2) and co-digestion (D3) digestates (230 and 245 mgCO₂ kg_{dm}⁻¹ d⁻¹, respectively). CO₂ emissions decreased steadily over time, reaching constant values similar to the control ones within 13 days. Conversely, the soil treated with unpretreated microalgae (D1) showed a different behaviour, whose highest value was observed after 2 days from the amendment (170 mgCO₂ kg_{dm}⁻¹ d⁻¹). Besides, cumulative net CO₂ emissions at the end of the incubation period increased in the following order: D1 < D3 < D2 (Table 4). Considering the amount of organic carbon added to the soil with the microalgae digestates (Table 4), higher fluxes of CO₂ were expected from D1 and D3 amended soils. However, the highest cumulative CO₂ emissions were detected for the soil amended with thermally pretreated microalgae, indicating that the organic matter of this digestate was less stabilised than the organic matter of the other digestates (D1 and D3). This is in accordance with the fact that D1 and D3 also showed lower biodegradability in the soil

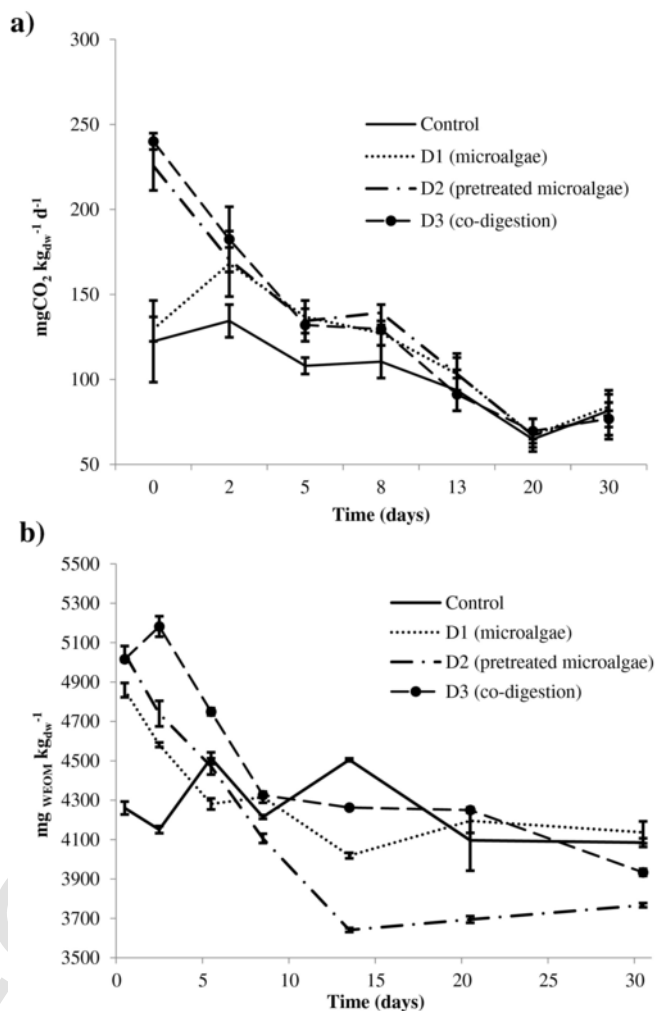


Fig. 2. (a) CO₂ emissions from microalgae-derived digestates amended soil (mean ± SD, n = 3); (b) Water extractable organic matter content in microalgae-derived digestates amended soil during the incubation period (mean ± SD, n = 3). Results are expressed on soil dry matter basis.

Table 4

Carbon mineralization rate from digestate amended soils after 30 days of incubation (mean ± SD, n = 3).

Parameter	Units	Digestate D1: microalgae	Digestate D2: pretreated microalgae	Digestate D3: co-digestion
Total N*	mg·L ⁻¹	2.4 ± 0.1	2.2 ± 0.1	1.9 ± 0.2
Application dose	mL	13.0	14.3	16.6
TOC _{added}	mg	98.1	92.0	101.1
WEOM	mg·L ⁻¹	1335.9	892.3	790.5
WEOM added	mg	17.4	12.8	13.1
Net CO ₂ emission	mg-C	21.2 ± 1.9	47.1 ± 2.1	30.7 ± 2.6
TOC _{added} mineralized	%	21.6 ± 1.7	51.2 ± 6.7	30.4 ± 5.2

Note: TOC = total organic carbon, WEOM = water extractable organic matter.

* Total N values used for the dosage calculation.

than D2. It can be deduced from the values of C-mineralization, expressed as the % of the added TOC that was mineralised at the end of the incubation (Table 4). The lower stabilisation of pretreated mi-

coalgae digestate with respect to the other digestates could be attributed to the different anaerobic digesters operations. For instance, comparing the anaerobic digestion of untreated and thermally pretreated microalgal biomass, higher NH_4^+ -N and VFA concentrations were found in the latter (Passos and Ferrer, 2014). As a consequence, the digestate from thermally pretreated microalgae could be less stabilised and could show higher soluble organic matter content that can be quickly mineralized in the soil. On the other hand, the co-digestion with primary sludge could also reduce the NH_4^+ -N and VFA concentrations in the reactors. The addition of easily degradable substances to the soil implies the consumption of soil oxygen that, in some circumstances, can lead to anoxic conditions, fermentation processes and to the production of phytotoxic substances (Wu et al., 2000). Stability-dependent respiration rates were reported by various authors for soils amended with organic materials (Sánchez-Monedero et al., 2004). Most of them also observed CO_2 emissions peaks in the first few days after amendment with an intensity related to the contents of WEOM and microbial biomass. In fact, it is well known that organic amendment can change the amount and quality of dissolved organic matter present in the soil solution (Chantigny, 2003). As WEOM is an easily available organic matter fraction for soil microorganisms, it has important implications on microbial activity and soil respiration. Moreover, Said-Pullicino et al. (2007) have shown that the soluble organic matter fraction of organic amendments tends to decrease with organic matter stabilisation.

Fig. 2b shows the time course of the WEOM in the digestate amended soils. Digestate application enhanced significantly ($P < 0.05$) the concentration of WEOM in the treated soils with respect to control during the first days after amendment. Following, the WEOM concentration showed a clear decreasing trend during the incubation period due to the soil microbial respiration. While D1 and D3 amended soils showed a decrease of WEOM content to the control level, in the D2 amended soils the WEOM mineralisation appears to be stronger and lead to a final content significantly lower ($P < 0.05$) than the control soils. The WEOM behaviour observed in the D2 amended soils and the low biodegradability showed by D1 and D3 appear to be in contrast with the WEOM concentrations in the microalgae-derived digestates (Table 4). In fact, D1 showed a higher content of WEOM with respect to D2 and D3. Therefore, it can be assumed that the labile organic matter of D2 was characterised by a low stability due to the thermal pretreatment of the microalgae biomass that was responsible for the solubilisation of labile and reactive organic compounds. As a consequence, the application of the thermal pretreated microalgae digestate to the soil can lead to the *priming effect*, with strong short-term changes in the turn-over of soil organic matter after the application of low stabilised organic amendments (Kuznyakov et al., 2000).

In all the amended soils, the strongest WEOM mineralization appeared to be concluded after 13 days from the application, similarly to what was observed for the CO_2 emissions. As already demonstrated by Pezzolla et al. (2013), when an organic amendment is applied to soil, WEOM is strictly related to the soil CO_2 emission rates. In the present work, this fact was confirmed by the correlation between the soil respiration rates of all the soil samples and their WEOM contents. Indeed, a high positive correlation was found ($y = 1.5313x - 2655.5$) to be significant ($r = 0.7750$) at $P < 0.05$ ($n = 28$). In the last two weeks of incubation a constant trend was observed for the WEOM content in the amended soils. This behaviour can be explained considering the dynamic equilibrium that occurs between the consumption of WEOM due to the mineralization and the release of WEOM by the soil microorganism during their hydrolytic activity (Rochette and Gregorich, 1998).

In the light of the results obtained, it appears clear that pretreated microalgae digestate is less recommendable for soil application than the other digestates due to the low stabilisation of its soluble organic matter. Indeed, untreated microalgae and co-digestion digestates spreading lead to a lower impact on soil system and higher benefits for the environment and the agriculture.

3.4. Evaluation of the potential phytotoxicity of digestates

Phytotoxicity effects are often found in anaerobic digestates due to the high contents in soluble salts, NH_4^+ -N and low weight organic compounds (i.e. volatile fatty acids, phenols) (Albuquerque et al., 2012). In this study, the GI was used to evaluate the digestates phytotoxicity by applying different concentrations of digestate (100%, 10%, 1% and 0.1%) and comparing the germination of cress seeds (*Lepidium sativum* L.) to a control (100% of deionised water) (Fig. 3).

The results showed that no germination was detected for any pure digestate. Thus, the GI of pure digestates (0%) indicates that they cannot be spread on agricultural soils without dilution or a stabilisation post-treatment process. For instance, a composting post-treatment would produce a compost where phytotoxic compounds, still abundant in anaerobic digestates and responsible of the absence of germination (Abdullahi et al., 2008), can be reduced. Conversely, positive results in the germination assays were found for digestate dilutions. Untreated and pretreated microalgae digestates (D1 and D2, respectively) gave a similar GI trend, showing the highest GI for the 0.1% dilution (109.9% and 97.3%, respectively). At this dilution (0.1%), the highest GI was observed for D1, probably due to the lower content of ammonia nitrogen with respect to D2 (Table 3). In both cases, the lowest GI value was observed at 10% dilution. On the contrary, no significant differences were observed between 1% and 0.1% dilutions, when values close to the control were achieved. It means that the largest phytotoxic potential was removed at 1% dilution. Concerning D3, there were no significant ($P < 0.05$) differences for the GI between dilutions of 10%, 1% and 0.1% (GI of 97.8%, 109.5% and 101.9% respectively), meaning that the phytotoxicity effect of the microalgae digestate was reduced through the co-digestion. Indeed, co-digestion processes are known to be more advantageous than mono-digestion ones due to a dilution effect of inhibitory compounds, among other factors (Tritt, 1992).

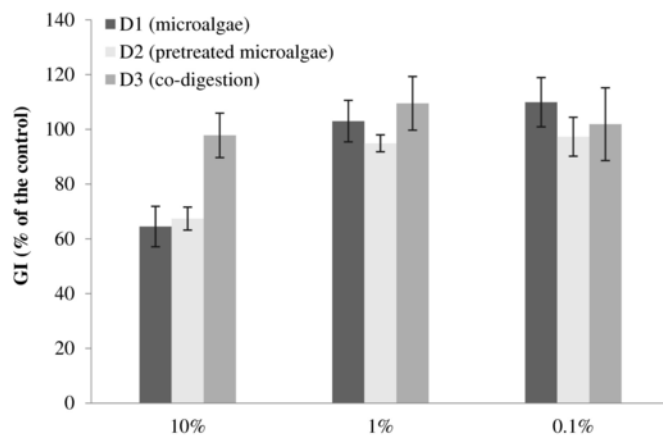


Fig. 3. Effects of microalgae digestates and their dilutions on the germination index (GI) of cress (*Lepidium sativum* L.) (mean + SD, $n = 5$). GI was 0% for all the pure (100%) digestates.

Moreover, the effect of digestates dilutions (10%, 1% and 0.1%) on the biomass production of cress (*Lepidium sativum* L.), expressed as GrI, were evaluated (Fig. 4). Concerning D1, no significant ($P < 0.05$) phytotoxic effect was detected on the production of biomass. Conversely, D2 showed a strong reduction of GrI at the highest concentration tested (10%), which is probably due to the high content of ammonium nitrogen of D2 (Table 3). At lower concentrations (1%, 0.1%), the GrI of D2 increased due to the dilution of the phytotoxic compounds. For both D1 and D2, the 1% dilution which showed a significantly higher ($P < 0.05$) GrI than the 0.1% dilution. As shown for other plants, low level of phytotoxicity can lead to a normal growth, or even higher than the un-stressed control, due to the genetic adaptability of the plants (Wang et al., 2015). This phenomena may be responsible of the GrI behaviours in D1 and D2. Nevertheless, the best performance in the plant growth bioassay was obtained from D3.

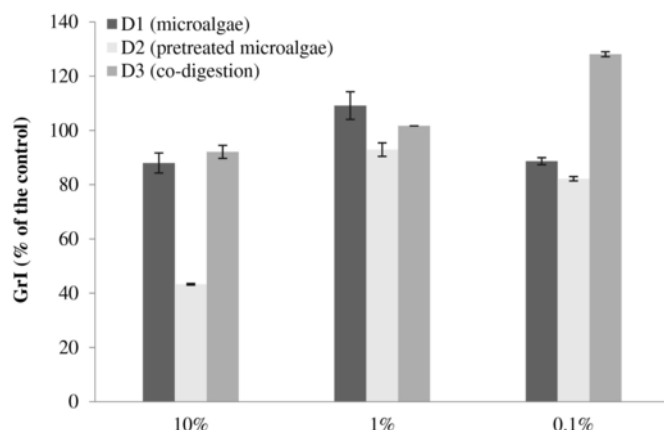


Fig. 4. Effects of microalgae digestates and their dilutions on the growth index (GrI) of cress (*Lepidium sativum* L.) (mean + SD, $n = 5$).

Table 5

Linear regression equations ($y = mx + q$) calculated for selected parameters of the digestates ($n = 11$).

Y	x	m	q	r
N-NH ₄ ⁺	GI	-0.0073	0.7254	0.9054*
VFA		-0.6728	67.351	0.9301*
EC		-0.0067	6.7041	0.9572*
N-NH ₄ ⁺	GrI	-0.0068	0.6826	0.8691*
VFA		-0.6270	63.0660	0.8862*
EC		-0.0628	6.2935	0.9156*

Note: GI = germination index, GrI = growth index, VFA = volatile fatty acids, EC = electric conductivity.

* Significant at $P < 0.05$.

Table 6

Concentration of heavy metals in microalgae digestates (mean + SD, $n = 3$).

Parameter	Units	Digestate D1: microalgae	Digestate D2: pretreated microalgae	Digestate D3: co-digestion	Limit values*	Limit values**
Cd	mg·kg TS ⁻¹	2.2 ^a ± 1.9	2.7 ^a ± 0.3	8.6 ^a ± 5.4	20–40	10
Cu	mg·kg TS ⁻¹	584 ^a ± 108	593 ^a ± 100	491 ^a ± 23	1000–1750	1000
Pb	mg·kg TS ⁻¹	47 ^a ± 3	49 ^a ± 1	221 ^b ± 112	750–1200	750
Zn	mg·kg TS ⁻¹	637 ^a ± 53	592 ^a ± 9	2202 ^b ± 135	2500–4000	2500
Ni	mg·kg TS ⁻¹	104 ^a ± 9	127 ^a ± 9	101 ^a ± 5	300–400	300
Cr	mg·kg TS ⁻¹	69 ^a ± 2	75 ^a ± 14	127 ^b ± 9	–	1000
Hg	mg·kg TS ⁻¹	2.0 ^a ± 0.5	1.7 ^a ± 0.6	< 1.1 ^a ± 0.2	16–25	10

a,b: Letters indicate a significant difference between digestates at the level of $p < 0.05$ after Tuckey's test.

* Limit values according to current European legislation (CEC, 1986)

** Limit values according to the European draft (CEC, 2003b)

Thus, co-digestion process appears to be the most suitable process for the reduction of phytotoxicity as already showed by the results obtained from the GI bioassay. Concerning the GrI determination, 10% and 1% dilutions of D3 did not show significant differences with respect to the control, showing the absence of residual phytotoxicity. When diluted at 0.1%, D3 showed plant nutrient, growth stimulant or even phytohormone-like effects (Albuquerque et al., 2012) that lead to a significant increase of the GrI ($P < 0.05$) with respect to the control (128.1%).

In the present work, NH₄⁺-N, VFA and EC of the digestates were found to be significantly ($P < 0.05$) and negatively correlated both to GI and GrI, as expected from what described in literature (Albuquerque et al., 2012; Zucconi et al., 1985). Statistical models used in this evaluation are described in Table 5.

In light of what was found in the germination and growth bioassays, agricultural application of the microalgae-derived digestates through dilution in the irrigation water would be the most suitable option, as the digestate would be diluted before coming in contact with seeds and plants. Moreover, dilution could also avoid salts and heavy metal concentration in the soil (Moral et al., 2005). Co-digestion digestate appeared to be the most suitable for agricultural reuse. In fact, it would require less water for dilution and, thus, it would be a more concentrated organic fertiliser. Moreover, the co-digestion digestate was the only one that did not show residual phytotoxicity; conversely it showed stimulating properties in the in vivo assays.

3.5. Potential risks of digestates: heavy metals and pathogens

In order to assess the potential risks of soil contamination after digestate spreading, the occurrence of heavy metals and the presence of pathogens (*E. coli*) were evaluated.

Concerning heavy metals, their concentrations in the three digestates were lower than the threshold established by the sludge European Directive (CEC, 1986), and also by the even more restrictive EU Directive draft (CEC, 2003b) (Table 6). Although all digestates presented appropriate heavy metal contents for soil application, special attention should be paid to the co-digestion digestate because of its high Zn content that is originated from the primary sludge. This is a particularity of the wastewater treatment plant where the primary sludge was collected, since they receive wastewater from industries generating high Zn concentration in their effluents. With regards to the microalgae digestate, despite microalgae ability for assimilating metals (Suresh Kumar et al., 2015), no significant heavy metal concentrations increase was found in microalgae digestates (D1 and D2) compared to the mixture with the primary sludge (D3) (Table 6).

Regarding the digestate hygienisation, low *E. coli* presence was found in all digestates (Table 7), below the threshold values proposed by the EU Directive draft on spreading sludge on land ($< 5 \cdot 10^5$ colony forming units per gram of wet weight of treated sludge) (CEC,

Table 7*Escherichia coli* content (CFU/ml) in microalgae digestates (mean \pm sd; n = 6).

Digestate	Mean	Maximum value
D1 (microalgae)	39.8	316.2
D2 (pretreated microalgae)	0.0	Absence
D3 (co-digestion)	25.1	199.5

Note: CFU = colony forming units.

2003b). Moreover, it is noteworthy that thermal pretreatment improved the hygienisation leading to absence of *E. coli* in the digestate. In fact, according to the EU draft, the combination of thermal pretreatment and anaerobic digestion can be considered as an advanced sludge treatment.

4. Conclusions

Agricultural reuse of the digestate from microalgae anaerobic digestion and co-digestion with primary sludge appears to be a promising solution towards zero waste generation in microalgae-based wastewater treatment systems. All microalgae digestates considered in this study presented organic matter and macronutrients content, especially organic and ammonium nitrogen, suitable for agricultural soils amendment. However, the thermal pretreated digestate presented a higher concentration of easily consumable organic carbon that can be mineralized on soil producing environmental impacts. Conversely, untreated microalgae and co-digestion digestates appeared to be more stabilised. In vivo bioassays demonstrated that the digestates did not show residual phytotoxicity when properly diluted, being the co-digestion digestate the one which presented less phytotoxicity. Furthermore, it showed interesting stimulant properties for plants. Heavy metals contents resulted far below the threshold established by the European legislation on sludge spreading. Low presence of *E. coli* was observed in all digestates. In addition, the thermal pretreatment improved the hygienisation obtaining absence of *E. coli* in the digestate. In this context, agricultural reuse of thermally pretreated microalgae and primary sludge co-digestate through irrigation emerges as a suitable strategy to recycle the nutrients and organic matter in agriculture.

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